Title page

Autotaxin, Bile Acid Profile and Effect of IBAT Inhibition in Primary Biliary Cholangitis Patients with Pruritus

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List of abbreviations

ALP, alkaline phosphatase; ALT, alanine transaminase, ANOVA, analysis of variance; ATX, autotaxin; BA, bile acid; BMI, body mass index; CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; FDR, false discovery rate; FGF19, fibroblast growth factor 19; GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; GDCA, glycodeoxycholic acid; GGT, gamma-glutamyl transferase; GUDCA, glycoursodeoxycholic acid; HC, healthy control; IBAT, ileal bile acid transporter; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine;

NMDS, non-metric multidimensional scaling; NRS, numerical rating scale; OTU operational taxonomic unit; PERMANOVA, permutational multivariate analysis of variance; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis; RCT, randomised controlled trial; rRNA, ribosomal RNA; TCA, taurocholic acid; TCDCA, taurochenodeoxycholic acid; TDCA, taurodeoxycholic acid; UDCA, ursodeoxycholic acid; UPLC-QToF-MS, ultraperformance liquid chromatography coupled to a quadrupole time-of-flight mass spectrometry;

Conflict of Interest

SK is an employee of GlaxoSmithKline (GSK). GMH and DEJ are investigators on the UK-PBC consortium which has received research funding from GSK. All other authors declare no competing interests related to the study.

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Abstract

Background &Aims: Pruritus is a common symptom in patients with primary biliary cholangitis (PBC) for which ileal bile acid transporter (IBAT) inhibition is emerging as a potential therapy. We explored the serum metabonome and gut microbiota profile in PBC patients with pruritus and investigated the effect of GSK2330672, an IBAT inhibitor.

Methods: We studied fasting serum bile acids (BAs), autotaxin and faecal microbiota in 22 PBC patients with pruritus at baseline and after two weeks of GSK2330672 treatment. Control group included 31 asymptomatic PBC patients and 18 healthy volunteers. BA profiling was done by ultraperformance liquid chromatography coupled to a mass spectrometry (UPLC-MS). Faecal microbiomes were analysed by 16S ribosomal RNA gene sequencing.

Results: In PBC patients with pruritus serum levels of total and glyco-conjugated primary BAs and autotaxin were significantly elevated. Autotaxin activity correlated significantly with tauro- and glyco-conjugated cholic acid (CA) and chenodeoxycholic acid (CDCA), both at baseline and after GSK2330672. GSK2330672 significantly reduced autotaxin and all tauro- and glyco-conjugated BAs and increased faecal levels of CA (p=0.048) and CDCA (p=0.027). Gut microbiota of PBC patients with pruritus was similar to control groups. GSK2330672 increased relative abundance of *Firmicutes* (p=0.033) and *Clostridia* (p=0.04) and decreased *Bacteroidetes* (p=0.033) and *Bacteroidia* (p=0.04).

Conclusions: Pruritus in PBC does not show a distinct gut bacterial profile but is associated with elevated serum bile acid and autotaxin levels which decrease after IBAT inhibition. In cholestatic pruritus, a complex interplay between BAs and ATX is likely and may be modified by IBAT inhibition.

Abstract word count: 250 words

Keywords: pruritus, PBC, metabonome, microbiota

Lay summary

- We compared serum bile acid, autotaxin and stool bacterial profile in PBC patients with and without itch and studied the effect of GSK2330672, a novel anti-pruritic drug.

- In PBC patients with itch elevated levels of bile acids and autotaxin were found without any significant difference in the gut bacterial composition.

- In PBC patients with itch GSK2330672 treatment decreased autotaxin and all major serum bile acids, increased faecal bile acids and changed gut bacterial composition.

- Bile acids and/or autotaxin may have role in itch associated with PBC and they may be modified by GSK2330672 treatment to improve itch.

Introduction

Primary biliary cholangitis (PBC) is a cholestatic liver disease, characterised by chronic inflammation and fibrotic destruction of interlobular bile ducts. If untreated, PBC may lead to biliary cirrhosis and need for liver transplantation.(1) Pruritus (itch) is a common and often a disabling symptom affecting up to 75% of patients at some point in their disease course.(2) It causes significant symptom burden and can produce a negative impact on health related quality of life.(2, 3) The anti-pruritic actions of bile acid (BA) sequestrants (e.g. colestyramine) point to potential role of BAs in the pathophysiology of cholestatic pruritus but the exact mechanism remains elusive.(4) Recent evidence shows serum autotaxin (ATX) activity is associated with cholestatic pruritus and its product lysophosphatidic acid (LPA) has been proposed as a candidate pruritogen in cholestasis.(5, 6) However, the relative contributions of ATX and total and individual BA species, and their mechanistic interactions in cholestatic pruritus remain obscure.

The treatment of pruritus in PBC is challenging due to the limited efficacy and poor tolerability of currently available drugs and lack of effective new therapies. Ileal bile acid transporter (IBAT) inhibitor agents are emerging as potential novel therapy for pruritus in PBC.(7-10) Recently, we investigated GSK2330672, a novel, selective human IBAT inhibitor in a phase 2a, randomised controlled trial (RCT) and showed that PBC patients with pruritus receiving two weeks of oral treatment with GSK2330672 had significant improvement in their pruritus compared to placebo.(11)

Over the years, metabonomics has been applied to study the metabolic signatures in a variety of liver diseases.(12) There are limited studies in cholestatic liver diseases with metabonomic profiling of serum/plasma and urine from patients with PBC and primary sclerosing cholangitis (PSC) and none of these studies specifically investigated pruritus associated with cholestasis.(13-16) Also, the effect of anti-pruritic therapy on metabolites associated cholestatic pruritus has had only preliminary exploration in published abstract reports on the effect of bezafibrate and albumin dialysis.(17, 18) Moreover, the effect of IBAT inhibitor on the metabolites associated with pruritus is currently unknown.

The role of gut microbiota in PBC is not clear. A recent study of patients with early-stage PBC reported alterations of the gut microbiome(19) and another study showed a distinct microbial diversity in ursodeoxycholic acid (UDCA)- treatment naïve PBC patients.(20) BAs modulate the gut microbiota and changes in intestinal BAs have been shown to significantly alter the composition of the gut microbiome in animal studies.(21) Also, the gut microbiota modulate the BA pool by metabolic deconjugation and transformation of primary BAs into secondary BAs.(22) Therefore, it is conceivable that in cholestatic pruritus, changes in BAs or microbiota or in the interaction of the two may have a role in the aetiology of the symptom, and may be modified by IBAT inhibition. However, to date, there are no studies reporting gut microbiota composition in patients with PBC and pruritus.

The main aim of this study was to characterise the serum metabolite profile and the faecal microbial composition in PBC patients with pruritus. We set out to test the following hypotheses:

1) PBC patients with pruritus have a distinct serum metabonomic signature and gut microbiome composition, compared to PBC patients without pruritus and/or healthy people; and

2) Pharmacological inhibition of enterohepatic circulation of BAs with an IBAT inhibitor can alter the serum and faecal BA profile, as well as change the faecal microbial composition in PBC patients with pruritus.

Materials and Methods

Participants

This prospective case-control study was carried out in two parts. In the first part, patients with PBC with pruritus were recruited to the BAT117213 study, a phase 2a, RCT of IBAT inhibitor GSK2330672. This RCT was sponsored by GlaxoSmithKline (GSK) and registered with EudraCT (2012-005531-84) and ClinicalTrials.gov (Identifier: NCT01899703). Ethical approval was given by the Research Ethics Committee NRES Committee North East – Sunderland (13/NE/0290). We recruited 22 PBC patients with pruritus between March 10, 2014, and Oct 7, 2015. Itch severity was assessed using a 0-10 numerical rating scale (NRS), PBC-40 itch domain score and 5-D itch scale.(23, 24) The trial protocol is available online (7) and we have recently published the safety and efficacy data of GSK2330672 in PBC patients with pruritus.(11)

In the second part, we recruited asymptomatic PBC patients (PBC-control) and healthy volunteers (HC). Participants in the PBC-control group were recruited only if they did not have any itch (assessed using PBC-40 itch domain score ≤ 3) and were not taking any anti-pruritic medications at the time of the study enrolment. Healthy volunteers who self-reported good health could enter the study when no known liver diseases were documented in their medical history. This study was sponsored by NIHR Newcastle BRC and approved by NRES Committee North East - Newcastle & North Tyneside 2 (14/NE/1036). PBC-control and HC were non-related, but were age (±2 years), gender and ethnicity matched to the PBC patients with pruritus of BAT117213 study.

The recruitment of participants in both studies occurred at two centres in the UK: Newcastle Upon Tyne Hospitals NHS Foundation Trust, Newcastle upon Tyne, and University Hospitals Birmingham NHS Foundation Trust, Birmingham. Informed consent was obtained from each patient included in the study and the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

Metabonomic analysis

The BA profiling analysis in faecal samples was performed using a 'semi-targeted' profiling method, utilizing an ultraperformance liquid chromatography coupled to a quadrupole time-of-flight mass spectrometry (UPLC-QToF-MS) assay at Imperial College London as previously reported.(25) In addition, quantitative measurements of up to 15 BAs in human serum was performed using Biocrates® Bile Acids Kit (BIOCRATES Life Sciences AG, Innsbruck, Austria). The assay was used with Waters Xevo® TQ MS triple quadrupole mass spectrometer (Waters Inc., Milford, Massachusetts, USA). Total bile acid (TBA) level was calculated by summation of 15 conjugated and unconjugated primary and secondary BA levels. We also used Biocrates Absolute*IDQ*® p150 kit (BIOCRATES Life Sciences AG, Innsbruck, Austria) with Waters TQ-MS to quantify acylcarnitines, amino acids, glycerophospholipids and sphingolipids. Serum ATX assay was quantified as recently described.(26) Serum fibroblast growth factor 19 (FGF19) was measured by a quantitative sandwich enzyme immunoassay technique according to the manufacturer's instructions (Human FGF19, Quantikine® ELISA, R&D Systems, Oxford, UK). ATX and FGF19 assays were conducted in the Academic Medical Centre, Amsterdam.

Metataxonomic analysis

We sequenced the V3-V4 region of the bacterial 16S ribosomal RNA (rRNA) gene to study the faecal bacterial composition in the study population. Sequencing was performed on the Illumina MiSeq platform (Illumina Inc., Saffron Walden, UK) using the MiSeq Reagent Kit v3 (Illumina) using paired-end 300bp chemistry. Further details of sample collection, preparation and statistical analysis of metabonomic and metataxonomic datasets are given in the **supplementary information**.

Results

We studied data from 22 PBC patients with pruritus, 31 PBC patients without pruritus (PBC-control) and 18 healthy volunteers (HC). None of the participants had taken any antibiotics for at least three months prior to study entry. The baseline demographic and clinical characteristics of the study groups are summarised in **Table 1**. The demographics and UDCA dose were comparable between PBC patients with pruritus and PBC-control. Serum levels of alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT) and bilirubin and PBC-40 itch domain scores were significantly higher in PBC patients with pruritus compared to PBC-control.

Bile acids and autotaxin in PBC with pruritus

PBC patients with pruritus had significantly elevated total BA level compared to PBC-control and HC with glycocholic acid (GCA) and glycochenodeoxycholic acid (GCDCA) levels significantly higher than PBC-control (**Table 2**). There was no difference in the levels of UDCA or its conjugates between PBC patients with pruritus and PBC-control.

In PBC patients with pruritus baseline serum ATX activity was significantly higher (**Figure 1A**) with mean serum level of total lysophosphatidylcholine (LPC) significantly lower (221±35.3 μM) compared to HC (259±47.3 μM, p=0.04) but not PBC-control (231±57.2 μM, p=0.72).

At baseline, 5-D itch scores significantly correlated with serum GCA (r=0.47, p=0.0257) and taurocholic acid [(TCA), r=0.45, p=0.0349] levels in PBC patients with pruritus (**table S1**). No significant correlations were seen between serum BAs, autotaxin and baseline PBC-40 itch domain score or NRS (**tables S2&S3**).

Analysis of other quantified serum metabolites showed significant differences in 43 metabolites between PBC patients with pruritus and HC (**table S4**). However, only one metabolite (C10:2, decadienylcarnitine) was significantly higher in PBC patients with pruritus (0.084±0.026 μM) compared to PBC-control (0.055±0.01 μM, p=0.013; Mann-Whitney test with FDR).

IBAT inhibition reduces bile acids and autotaxin

Serum and faecal BA profile data for pre- and post-GSK2330672 were available for 16 patients (samples from six patients were insufficient for analysis). Compared to the baseline, two weeks of GSK2330672 treatment significantly reduced serum levels of all tauro- and glyco-conjugated BAs (**Table 3**). Total BA level also decreased, but did not reach statistical significance (p=0.057). Serum levels of chenodeoxycholic acid (CDCA) and deoxycholic acid (DCA) significantly increased but cholic acid (CA) did not change significantly (p=0.78). GSK2330672 treatment significantly decreased serum ATX activity levels (**Figure 1B**).

Faecal BA profiling (n=14) showed significantly increased levels of total BA, CA, CDCA and DCA following GSK2330672 treatment, compared to the baseline (**Figure 2**). No significant differences were seen in other conjugated primary or secondary BAs (**figure S1**).

Compared to the baseline, no significant changes were seen in other measured serum metabolites including acylcarnitines, glycerophospholipids or sphingolipids following GSK2330672 (data not shown).

Serum autotaxin correlates with bile acids

In PBC patients with pruritus significant correlations were observed between conjugated primary and secondary BA levels and serum ATX activity at baseline (**Table 4**). Also, following GSK2330672 treatment percentage (%) changes (Δ) in serum ATX activity from baseline correlated significantly with %Δ in serum BA levels from baseline (**Table 4**). However, %Δ in serum BAs (total or individual) or ATX activity did not significantly correlate with %Δ in 5-D itch, PBC-40 itch domain or NRS cores (**tables S5-7**).

Gut bacterial profile in PBC with pruritus

The faecal bacterial composition of PBC patients with pruritus was not significantly different from the two control cohorts. Compositional analysis performed on phylum, class and order levels showed relative abundance of faecal bacteria from PBC patients with pruritus was not significantly different from those of PBC-control or HC ($p>0.05$ for all comparisons, ANOVA with Benjamini Hochberg FDR). Comparison of diversity indices showed no significant differences in the Chao1 index ($p=0.051$, Kruskal-Wallis test) or Shannon index ($p=0.923$, Kruskal-Wallis test) between study cohorts (**figure S2**).

IBAT inhibition alters gut bacterial profile

Gut bacterial composition of PBC patients with pruritus was compared at baseline and after 14 days of treatment with GSK2330672 or placebo. For each subject, relative abundance of operational taxonomic units (OTUs) determined at the phylum level is shown in **figure S3.** A non-metric multidimensional scaling (NMDS) plot showed clear separation of bacterial composition after GSK2330672 treatment (**figure S4A**). Overall, GSK2330672 significantly

changed the bacterial community composition at the phylum level (PERMANOVA p=0.027), with a significant decrease in *Bacteroidetes* (p=0.033) and increase in *Firmicutes* (p=0.033) (**figure S4B)**. Significant changes were also seen at the class and order levels with decrease in *Bacteroidia* (p=0.040) and *Bacteroidales* (p=0.011) and increase in *Clostridia* (p=0.040) and *Clostridiales* (p=0.044), respectively (**figure S4C&D)**. No significant changes were seen at other taxonomic levels.

Changes in faecal microbiota and faecal BA levels following GSK2330672 correlated with strongly positive correlation seen between phylum *Firmicutes* and CA (r=0.99) and CDCA (r=0.95) and negative correlation between phylum *Bacteroidetes* and CA (r=-0.74) and CDCA (r=-0.68) (**figure S5**).

Discussion

We report the serum metabonomic profile and gut bacterial composition in PBC patients with pruritus and describe the effects of IBAT inhibition on serum and faecal BAs and compositional alterations in faecal bacteria in this patient group.

In this study, we found altered serum BA profile in PBC patients with pruritus compared to PBC patients without pruritus. In addition to significantly higher levels of total BA, GCA and GCDCA, we observed GCA and TCA correlated with 5-D itch scores in PBC patients with pruritus. A recent trial of NGM282 (an engineered analogue of FGF19) also found significant association between baseline 5-D itch scores and serum GCA and TCA in patients with PBC.(27) We acknowledge that our cohort of PBC patients with pruritus had higher baseline levels of ALP, GGT and bilirubin but FGF19 levels were similar to PBC patients without pruritus. Since serum FGF19 levels are linked to the severity of cholestasis,(28) the latter is unlikely to have biased our serum BA results.

We observed significant decrease in serum total and conjugated BAs following pharmacological IBAT inhibition with GSK2330672. In addition, we have recently reported that GSK2330672 treatment significantly improved pruritus scores in PBC patients with pruritus.(11) Therefore, the anti-pruritic effect of an IBAT inhibitor agent could be mediated by reduction in circulating BAs. However, reductions in serum BAs did not correlate with reductions in pruritus scores. In a historic study fasting total BA levels were found to be higher in patients with pruritus compared to those without pruritus.(29) A positive relationship between pruritus and serum BAs has been shown(30) and improvement in pruritus with BA binding resin cholestyramine further supports

their role.(31) Taken together, our findings on differential BAs in PBC patients with pruritus and changes after IBAT inhibition therapy may suggest that serum (total or individual) BAs may have pathogenetic role in cholestatic pruritus.

We also studied serum ATX which drives enzymatic conversion of LPC into LPA, a novel proposed pruritogen in cholestatic diseases.(6, 32) Similar to previous studies, we found elevated serum ATX activity in PBC patients with pruritus. Interestingly, we also observed correlations between serum BAs and ATX activity at baseline, with a strong correlation between GCDCA and ATX ($r=0.80$, $p<0.0001$). Also, reductions in tauro- and glyco-conjugated primary BAs and ATX levels after GSK2330672 treatment correlated significantly. Our observations on association between serum BAs and ATX are novel. This, in addition to the recent intriguing finding of the inhibitory effect of GCDCA on ATX activity (33) merits further investigation into the complex interplay between BAs and ATX in cholestatic pruritus.

In the current literature there are only two reports on intestinal microbiota composition in PBC. In their study Lv *et al*. observed early stage PBC patients had reductions of several potentially beneficial gut microbiota (such as Acidobacteria, *Lachnobacterium* sp., etc.), and the enrichment of some opportunistic pathogens (such as γ-Proteobacteria, Enterobacteriaceae, etc.).(19) Tang and co-workers observed reduced species richness and a lower level of microbial diversity in patients with PBC and partial restoration of these changes after UDCA treatment.(20) However, these investigators did not report gut microbiota in relation to pruritus associated with PBC. We hypothesized that pruritus in PBC is associated with specific gut bacterial dysbiosis. But our results did not show any significant difference in faecal bacterial composition or diversity

between PBC patients with pruritus compared to the control group. This lack of difference may suggest that cholestatic pruritus may not be associated with a specific gut bacterial composition. However, since we did not study functional alterations in the gut microbiota we cannot exclude the possibility of microbial metabolites contributing to cholestatic pruritus. Therefore, our negative findings on gut microbiota need to be confirmed in larger studies and additional studies are needed to investigate the role of gut microbial metabolites in cholestatic pruritus.

Evidence suggest that BAs are important in regulating gut microbial community structure (34, 35) and animal data show regulatory effects of gut microbiota on BA homeostasis.(36, 37) Although effects of IBAT inhibitor agents on serum and faecal BA levels have been studied in animal models of cholestasis,(38, 39) to date, there are no human studies on the effect of IBAT inhibition on the gut microbiota. We observed that in PBC patients with pruritus treated with an IBAT inhibitor agent faecal BA levels increased and faecal bacterial composition significantly changed from baseline. Increased faecal DCA levels could indicate increased conversion of CA to DCA by gut microbiota derived 7-α-dehydroxylase enzymes. Major taxonomic alterations were seen at the phylum, class and order-levels respectively, with significant decreases in *Bacteroidetes*, *Bacteroidia* and *Bacteroidales* and increases in *Firmicutes*, *Clostridia* and *Clostridiales*. We hypothesize that these changes are at least in part due to the direct effect of increased BA load in the colon resulting from IBAT inhibition. This idea is supported by increased faecal CA and CDCA levels after GSK2330672 and their strong correlations with *Firmicutes* and *Bacteroidetes*. Interestingly, our findings are similar to Islam and colleagues study, where rats fed with high CA diet showed significant expansions in *Firmicutes* (from 54%

to 93-98%) and *Clostridia* (from 39% to 70%) and significant inhibition of the *Bacteroidetes*.(21) However, an important question that remains unanswered by our study, but that merits further investigation is, whether the changes in the gut microbiome produced by the IBAT inhibitor contribute to its anti-pruritic effect in PBC via changes in faecal microbial metabolites.

Although we have attempted to provide a comprehensive insight into the serum metabonome and gut microbiota in cholestatic pruritus, our study has limitations to be addressed in future studies. First, our relatively small cohort may have resulted in insufficient statistical power to unravel all metabolic perturbations. To determine the complete metabonome profile and microbial diversities, a large cohort of PBC patients with pruritus is required. Ongoing clinical development of GSK2330672 (NCT02966834) may present the opportunity for further study of metabonomic and microbiomic profile in cholestatic pruritus. Second, we did not investigate the metagenome (functional composition profile) of microbiota which may help in analysis of pathway(s) associated with cholestatic pruritus. Third, instead of mucosal microbiota we opted to study stool samples, but it is known that faecal bacterial profiles do not fully replicate mucosa associated profiles.(40) Also, we did not objectively assess stool consistency, which is recently shown to be strongly associated with gut microbiota composition.(41) Finally, although our cohort was matched for age, BMI and ethnicity, results could be influenced by other confounding effects such as environment and dietary factors.

In summary, in PBC patients with pruritus we observed elevated serum bile acid and autotaxin levels which decreased after anti-pruritic treatment with an IBAT inhibitor agent. The strong

correlation between serum bile acids and autotaxin at baseline and post IBAT inhibition suggests a complex interplay between bile acids and autotaxin in cholestatic pruritus is likely and may be modified by IBAT inhibition to reduce pruritus. Gut bacterial composition of PBC patients with pruritus was not different from control but altered significantly following IBAT inhibition. Our findings need to be confirmed in future studies which should focus on further dissecting the underlying molecular mechanism of cholestatic pruritus and clarifying the mechanisms of the anti-pruritic effect of IBAT inhibitor agents.

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Figure legends

Figure 1 Serum autotaxin activity **A**) in study cohorts, and **B**) in PBC patients with pruritus at baseline and after treatment with GSK2330672. (Data in mean±SD; Unpaired t-test used in figure 1A and paired t-test in figure 1B).

Figure 2 Faecal bile acid profile in PBC patients with pruritus at baseline and after treatment with GSK2330672. **A**) Total and **B**) individual bile acids. (Data in mean±SD. *P* values adjusted with FDR correction as described in method section).